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The balance between proliferation and transcription of angiogenic factors of mesenchymal stem cells in hypoxia

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ABSTRACT

Bridging large bone defects with mesenchymal stromal cells-seeded scaffolds remains a big challenge in orthopedic surgery, due to the lack of vascularization. Within such a cell–scaffold construct, cells are exposed to ischemic conditions. When human mesenchymal stem cells (hMSCs) encounter hypoxic conditions, they show higher cell proliferation than at ambient oxygen levels. However, when hMSCs are exposed to prolonged ischemia, cell proliferation ceases completely. Exposure of hMSCs to hypoxic conditions is known to result in the transcription of angiogenic factors (AGF), which can promote the development of new blood vessels. In this study, we investigated at which oxygen level hMSC proliferation and the transcription of AGF were optimal. Human bone marrow-derived hMSCs were cultured at 0.1, 1, 2, 3, 4, 5, and 21% oxygen. Cell proliferation over 14 days was assayed using a DNA quantification method. hMSC metabolic activity over 14 days was measured using a MTT test. Quantitative RT-PCR was used to assess mRNA levels of angiogenic factors at the tested oxygen percentages. hMSCs showed the highest cell proliferation rate at 1% oxygen. The highest corrected cell metabolic rate was found at 21% oxygen, followed by 2% oxygen. HIF1α transcription did not increase under hypoxic conditions compared to 21% oxygen conditions. However, transcription of VEGF and ANG-1 was significantly higher at 2% oxygen than at 21% O_2. The optimum oxygen range at which hMSCs proliferated rapidly and angiogenic factors ANG-1 and VEGF simultaneously came to expression was from 1 to 2% oxygen.

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Angiogenic factor; cell proliferation; human mesenchymal stem cells; hypoxia; oxygen

Introduction

One of the biggest challenges in the regeneration of bone is the establishment of a vascular network in large cell-seeded scaffolds. Due to the lack of a vascular network at implantation, cells in the center of a large scaffold are deprived of sufficient oxygen (1,2). In particular, in poorly vascularized tissue, as is the case with bone, sufficient vascular ingrowth is hard to achieve. Moreover, the scaffold is implanted in a bone defect site that usually already has impaired vascularization due to disruption of bone and periost integrity. These two conditions expose the cells seeded on a scaffold to an unfavorable, anoxic environment immediately after implantation in bone.

For transport over distances larger than several millimeters within the body, oxygen is mainly bound to hemoglobin. When bound to hemoglobin in erythrocytes, oxygen is transported rapidly and efficiently over large distances to vascularized tissues. After having been released from hemoglobin, oxygen is transported to the cells through diffusion. Diffusion is a relatively slow process, and oxygen can travel only short distances (3). The maximum diffusion distance of oxygen within the body is 100–200 μm (4). The presence of a vascular network in a cell–scaffold complex is therefore essential for sufficient oxygen provision to the cells. Several methods to improve vascular supply within tissue-engineered scaffolds have been studied, such as application of vascular growth factors on scaffolds to stimulate vascular ingrowth after implantation, design of scaffolds with micro channels that mimic blood vessel structures, and preculturing of cell-seeded scaffolds in bioreactors, so that growth of a vascular network can be initiated prior to implantation of a scaffold (1,5). Still, many techniques aiming at improvement in vascularization within large, artificial cells–scaffold complexes, require vascular ingrowth after implantation of the scaffold.

Vascular ingrowth is stimulated by angiogenic factors (AGFs). These are proteins that promote the sprouting of new blood vessels from the already existing vascular network in the near surroundings. AGFs

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are produced endogenously in response to among others, hypoxia, low pH, hypoglycemia, and an inflammatory reaction (4). The central factor that regulates the production of AGFs in response to hypoxia is hypoxia induced factor 1α (HIF-1α) (Figure 1). HIF-1α is transcribed, produced, and metabolized continuously in all human cells, but HIF-1α degradation is slowed down under hypoxic conditions. This "angiogenic cascade" starts with the coupling of HIF-1α to HIF-1β in the nuclei of human cells (6). The HIF complex binds to the hypoxia-responsive element (HRE) in the promoters of the genes of the AGFs, initiating transcription (6). Subsequently, several AGFs will be synthesized that stimulate several phases of vascular growth, such as increasing vascular permeability (VEGF, FGF), migration and proliferation of endothelial cells (VEGF-*R*2), and maturation of blood vessels (TGF-β1, ANG-1, and PDGF-β) (7). Whenever oxygen supply is restored, HIF-1α will be metabolized again; therefore, the angiogenic cascade can no longer be initiated, and cells will cease the transcription of AGF genes. Whenever oxygen supply is demolished, cell metabolism decreases and cell death occurs (8). Obviously, the transcription of AGF will be lowered and eventually stop as well. We therefore hypothesize that AGF expression plotted against oxygen level results in a bell-shaped curve; at very low oxygen conditions, AGF expression is absent or very low, and at low oxygen conditions, AGF expression rises and in sufficient oxygen conditions AGF expression descends again. It is not yet known at which oxygen levels exactly AGF expression increases and decreases.

Human mesenchymal "stem" cells (hMSCs) show great potential as the cell component for bone regeneration. These cells can easily be isolated from various types of tissue, differentiate into several cell types and have trophic effects (9,10). A frequently used source of hMSCs is bone marrow. Naturally, bone marrow-derived hMSCs (BM-hMSCs) reside in the niche areas of bone marrow, where the standard partial oxygen level is 1–2% (11,12), which equals 1.0–7.1 kPa or 7.5–53.2 mmHg oxygen. However, in cell culture studies, BM-hMSCs are often cultured at ambient oxygen level (21%), which is a hyperoxic situation for the cells. For standardization purposes, any oxygen percentage below 21% will be referred to as hypoxia in this study and 21% as normoxia. Results of previous studies show that BM-hMSCs tend to proliferate faster at hypoxic oxygen levels than at 21% oxygen (13–16). At very low oxygen conditions of 1% O_2 and lower, a decreased proliferation rate has been reported (17,18). In conclusion, the curve of BM-hMSC proliferation plotted against oxygen level seems to have a bell-shaped curve too; at very low oxygen levels, cell proliferation is low, until cell death occurs at severe hypoxia (8), while a slight increase in oxygen tension until a certain level results in higher cell proliferation. It is not yet known at which oxygen levels exactly BM-hMSC proliferation increases and decreases. For hMSCs derived from tissue types other than bone marrow, the oxygen levels at which cell proliferation increases and decreases may be different.

We hypothesize that if an overlay of the AGF expression curve and the cell proliferation curve were created, there would be a window in oxygen level at which BM-hMSCs cultured under hypoxia show increased AGF expression and still proliferate. If BM-hMSCs are cultured at oxygen levels within this window, optimal proliferation and AGF expression may be accomplished, and this window may serve as a basis for further clinical research. This study therefore aims at finding the oxygen concentration range at which hMSCs both proliferate and induce vascular ingrowth through expression of AGF.

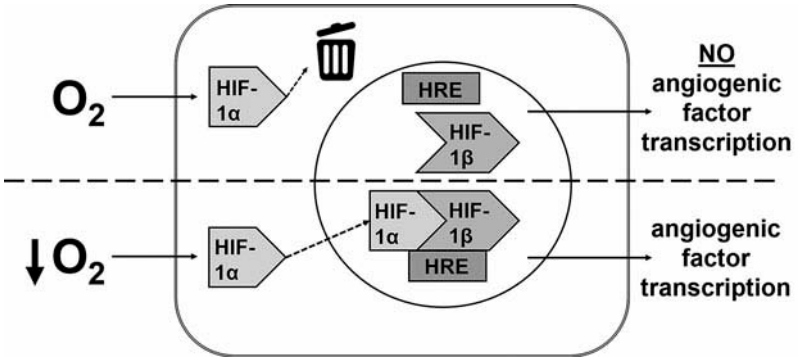

Figure 1. Schematic representation of the initiation of the angiogenic cascade in a cell. Whenever sufficient oxygen is supplied to the cell (upper half of figure), HIF-1α is metabolized in the cytoplasm, and the angiogenic cascade is not started. Whenever insufficient oxygen is supplied to the cell (lower half of the picture), HIF-1α proceeds to the cell nucleus and binds to HIF-1β. The HIF-1α-HIF-1β-complex binds to the HRE, and angiogenic factor transcription is initiated.

Materials and methods

Isolation and culture of MSCs

Reaming debris from the femur acquired during total hip replacement surgeries was used as a source of bone marrow. All three donors were females, aged 55, 71 and 85. The ethics committee of University Medical Center Groningen approved the study protocol.

From each patient, 12 ml of bone marrow was aspirated using a Jamshidi bone marrow aspiration needle (Beckton Dickinson, Franklin Lakes, NJ) and divided into four equal portions. The bone marrow portions were diluted with phosphate-buffered saline (PBS), and subsequently, density gradient centrifugation (Histopaque 1077, Sigma-Aldrich, Steinheim, Germany) was done according to the manufacturer's instructions. The mononuclear cell fraction was seeded in T75 tissue culture flasks (Greiner Bio-one, Alphen aan den Rijn, the Netherlands). A standard medium, consisting of minimal essential medium-alpha (α-MEM, Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 0.1 mM Ascorbic Acid-2-Phosphate (AA2P, Fluka, Steinheim, Germany) and 2% antibiotic–antimycotic (Invitrogen) was added, and the cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was changed twice weekly. When 70% confluence was reached, the obtained cells were suspended in a full culture medium containing 7.5% DMSO (Sigma-Aldrich), frozen, and stored in liquid nitrogen. For each oxygen percentage tested, a new batch of cells was thawed and grown up to 70% confluence prior to the experiment.

Characterization of MSCs

For characterization, hMSCs were thawed and cultured in T75 tissue culture flasks with standard medium in a humidified atmosphere with 5% CO_2 at 37 °C until passage 3. hMSCs from each individual donor were tested in triplicate.

To assess the antigen expression profile of the isolated cells, a standard set of antibodies was conjugated to the cells (BD Stemflow Human MSC Analysis Kit, BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Fluorescent cells were counted using a FACS machine (FACSCalibur, Beckton Dickinson, Franklin Lakes, NJ). The Kaluza software package (Beckman Coulter, Brea, CA) was used to analyze the FACS data.

The cells isolated from bone marrow were differentiated into osteogenic, adipogenic, and chondrogenic lineages. To assess osteogenic potential, 3750 cells/cm^2 were cultured in a 24-well tissue culture plate (Greiner Bio-one) with osteogenic medium [α-MEM, 2% antibiotic–antimycotic, 10% heat-inactivated FBS, 100 nM dexamethasone (Sigma-Aldrich), 0.05 mM β-glycerophosphate (Sigma-Aldrich) and 50 μM AA2P] for two weeks. Thereafter, alkaline phosphatase production in the cells was assayed using an alkaline phosphatase assay (Leukocyte alkaline phosphatase kit; Sigma) according to the manufacturer's instructions. To assess adipogenic potential, 5 000 cells/cm^2 were cultured in a 24-well tissue culture plate with adipogenic medium [α-MEM, 2% antibiotic–antimycotic, 10% heat inactivated FBS, 1 μM dexamethasone, 0.5 mM IBMX (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), and 10 μM human insulin (Invitrogen)] for three weeks. After three weeks, the cells were fixated using 3.7% paraformaldehyde (Boom, Meppel, the Netherlands). Subsequently, the cells were stained using a 0.3% (w/v) Oil Red O (Sigma-Aldrich) in 99% 2-propanol solution, diluted 1.67 times in water.

To assess chondrogenic potential, 250,000 cells were seeded in 15-ml conical tubes and spun down in a centrifuge. The cell pellet thus created was cultured with chondrogenic medium [α-MEM, 2% antibiotic–antimycotic, 0.1 mM AA2P, 0.1 μM dexamethasone, 1% ITS+ (Invitrogen), 40 μg/ml L-proline (Sigma-Aldrich) and 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN)] for two weeks. Next, the cell pellets were fixed with paraformaldehyde (3.7%), dehydrated in a series of increasing concentration ethanol solutions, and embedded in paraffin. Five micrometer sections were stained using 1% alcian blue (Boom) in 0.1 M HCl solution and counterstained with 0.2% (w/v) nuclear fast red (Merck, Darmstadt, Germany) staining solution.

Table 1. Primer sequences.

Primer name	Sequences
B2M	Forward5′-TGGCGGGCATTCCTGAAGCTGACA-3′ Reverse 5′-CAGCTAAGGCCACGGAGCGAGACA-3′
RPL13a	Forward5′-GAGGTATGCTGCCCCACAAA-3′ Reverse 5′-GTGGGATGCCGTCAAACAC-3′
ANG-1	Forward5′-GGTGTTTTACTAAAGGGAGGAA-3′ Reverse 5′-TTGCAAAACACCTTTTTGGG-3′
FGF-2	Forward5′-GGGAGAAGAGCGACCCTCACATCAA-3′ Reverse 5′-AGCCAGGTAACGGTTAGCACACACT-3′
HIF-1α	Forward5′-CGTTCCTTCGATCAGTTGTC-3′ Reverse 5′-TCAGTGGTGGCAGTGGTAGT-3′
PDGF-β	Forward5′-CTCTTCCTGTCTCTCTGCT-3′ Reverse 5′-ATCTTCCTCTCCGGGGTC-3′
TGF-β1	Forward5′-AACAATTCCTGGCGATACCT-3′ Reverse 5′-GTAGTGAACCCGTTGATGTC-3′
VEGF	Forward5′-CCTGGTGGACATCTTCCAGGAGTACC-3′ Reverse 5′-GAAGCTCATCTCTCCTATGTGCTGGC-3′
VEGF-*R2*	Forward5′-CTGGTCTTTTGGTGTTTTGC-3′ Reverse 5′-TGGTCTGGTACATTTCTGGT-3′

Cell proliferation

hMSCs from each individual donor were seeded eightfold in a 96-wells plate (Greiner Bio-one), at passage 3

and at a density of 3,125 cells/cm^2. The cells were cultured with 100 µl/well standard medium without antibiotic–antimycotic in a hypoxic cabinet (Invivo$_{200}$, Ruskinn, Glamorgan, UK) at gas mixtures containing 5% CO_2 and 0.1, 1, 2, 3, 4, or 5% O_2, at 37 °C and 100% humidity. As a control, cells cultured at 21% O_2, 5% CO_2, and at 37 °C were used. After 1, 3, 7, 10, and 14 days of incubation, the cell layers were briefly rinsed with PBS once and frozen at -80 °C. At the end of the experiment, the cells were thawed, and the amount of DNA per well was quantified using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's instructions. A DNA standard curve was made using lambda DNA, according to the manufacturer's instructions.

Cell metabolic rate

Cell metabolic rate was assessed using a (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide/thiazolyl blue tetrazolium bromide (MTT) assay. In a 24-wells plate, hMSCs from each individual donor were seeded at a density of 5,000 cells/cm^2 in triplicate and incubated with 1 ml standard medium without antibiotic–antimycotic per well at 0.1, 1, 2, 3, 4, or 5% O_2 in a hypoxic cabinet. Cells were used for the experiments at passage 3. After 1, 3, 7, 10, and 14 days of incubation, the cells were washed with PBS once and full cell culture medium supplemented with 0.5 mg/ml of thiazolyl blue tetrazolium bromide (Sigma Aldrich) was added. The cells were incubated with the MTT medium for 2.5 hours in the hypoxic cabinet. After incubation, cells were carefully washed with PBS once, and 500 µl of 2-propanol (Merck) was added to each well. The plates were placed on a shaker for two minutes, and the 2-propanol supernatants were subsequently pipetted into microcentrifuge vials and stored at 4 °C. When a time range at a specific O_2 percentage was completed, the supernatants were centrifuged for 30 seconds at maximum speed. The supernatants (100 µl) were pipetted in a 96-well plate in triplicate, and the absorbance was read at 570 nm using a plate reader (Fluostar Optima, BMG labtech, Olfenburg, Germany).

The cell metabolic rate was corrected for the number of cells per condition and per time point using Equation (1):

$$Corrected\ Cell\ Metabolic\ Rate\ (CCMR) = \frac{Average\ MTT\ value\ (per\ time\ point\ and\ per\ oxygen\ percentage)}{Average\ number\ of\ cells\ per\ well\ (per\ time\ point\ and\ per\ oxygen\ percentage)} \quad (1)$$

Angiogenic factor expression

For RNA isolation, hMSCs from each individual donor were seeded at a density of 11,000 cells/cm^2 in 6-well plates (Greiner Bio-one) and cultured with 3 ml of standard medium without antibiotic–antimycotic at 21% O_2 for two days. Cells were seeded at passage 3. The experiments were performed in triplicate. After two days of culture, the cells were placed in a hypoxic cabinet and maintained at gas mixtures containing 5% CO_2 and 0.1, 1, 2, 3, 4, or 5% O_2, at 37 °C and 100% humidity for six hours. Subsequently, the cells were lysed using RLT buffer (RNeasy Micro Kit, Qiagen, Hilden, Germany), and the lysates were stored at -80 °C until RNA was isolated. RNA was isolated using a RNeasy Micro Kit according to the manufacturer's instructions. As a control, cells cultured at 21% O_2 and 5% CO_2 and at 37 °C were used. The amount of isolated RNA was measured using spectrophotometry (NanoDrop 1000, NanoDrop, Wilmington, DE). Copy DNA (cDNA) was made out of 200 ng RNA using a DNA polymerase protocol (iScript cDNA Synthesis Kit, BioRad, Hercules, CA) according to the manufacturer's instructions. The 10-times diluted cDNA was stored at -80 °C. qPCR was done using the iQ™ SYBR® Green Supermix kit (BioRad, Hercules, CA) according to the manufacturer's instructions on a PCR machine (CFX384 Real Time System, BioRad). hRPL13a and B2M were used as reference genes. Several AGFs were assayed; primer sequences are given in Table 1. For calculation of expression levels of all genes, the Livak method was used (19).

Statistical analysis

SPSS 20.0 was used for all statistical analyses. Data were tested for normality, which could not be confirmed in all tests. As outcomes of more than two oxygen percentages were compared, Kruskal–Wallis tests were used for data analysis. A p value of 0.05 or lower was considered to be significant. A p value of > 0.05 and < 0.10 was considered a trend.

Results

Human mesenchymal stem cells

The cell populations obtained from the three donors met the criteria as stated by the International Society of Cellular Therapy (ISCT) (20). The cells were selected from the nucleated cell fraction of the bone marrow samples by their ability to adhere to tissue culture plastic. The antigen expression profile as assayed using FACS met the ISCT criteria. No differences in antigen expression profile between donors could be found. The differentiation potential in adipogenic, chondrogenic,

and osteogenic cell lineages was confirmed through comparison with previous literature (21).

Cell proliferation

hMSC proliferation was comparable at most oxygen percentages, except when cells were cultured at 1% O_2 (Figure 2). Already on the first day after cell seeding, cell proliferation was significantly higher in cells cultured at 1% O_2 than at most other oxygen percentages (*p* values shown in Table 2). The lowest oxygen percentage that was tested, 0.1%, resulted in significantly lower cell proliferation than most other oxygen percentages from day 7 onwards. Cells cultured at 21% O_2 rapidly proliferated in the first three days after seeding. On day 4, cell proliferation at 21% O_2 decreased. At day 14, the cell proliferation was significantly lower at 21% oxygen than at 1, 2, 3, and 4% O_2.

Cell metabolic activity

The corrected cell metabolic rate (CCMR) was significantly higher in cells cultured at 21% O_2 than in cells cultured at most other oxygen percentages after day 3 (see Figure 3, *p* values are shown in Table 3). When comparing the CCMR at 21% and 0.1% oxygen, there was a trend toward a significant difference. The second highest CCMR was found in cells cultured at 2% oxygen, but the CCMRs were similar at all oxygen percentages other than 21% O_2.

Angiogenic factor expression

Both B2M and RPL13a were used as reference primers to calculate the normalized fold expression of several AGF. Gene expression was calculated using each reference primer and both gave similar results. Only the normalized fold expression as referred to RPL13a expression is shown here. The reference condition used was gene expression at 21% oxygen (set at 1 in Figure 4). The expression of vascular endothelial growth factor (VEGF) was highest

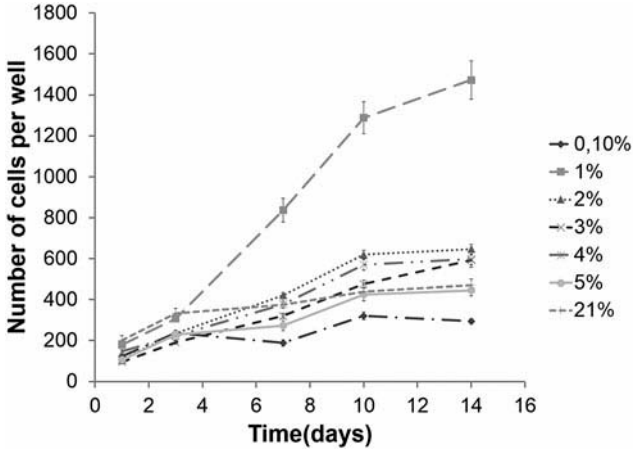

Figure 2. Line graph representing the number of cells per well at several oxygen percentages measured at five time points over 14 days. Error bars represent the standard error of the mean.

Table 2. Table indicating *p* values of differences in cell proliferation of 0.1, 1, and 21% oxygen as compared to all other tested oxygen percentages, for five time points.

		Time (days)				
O_2 percentage (%)	Compared to (%)	1	3	7	10	14
0.1	1	0.00*	0.06	0.00*	0.00*	0.00*
0.1	2	0.13	0.88	0.00*	0.00*	0.00*
0.1	3	0.18	0.01*	0.00*	0.00*	0.00*
0.1	4	0.16	0.88	0.00*	0.00*	0.00*
0.1	5	0.18	0.41	0.02*	0.08	0.00*
0.1	21	0.21	0.08	0.00*	0.00*	0.00*
1	2	0.00*	0.00*	0.00*	0.00*	0.00*
1	3	0.00*	0.00*	0.00*	0.00*	0.00*
1	4	0.00*	0.00*	0.00*	0.00*	0.00*
1	5	0.00*	0.00*	0.00*	0.00*	0.00*
1	21	0.54	0.58	0.00*	0.00*	0.00*
21	2	0.02*	0.00*	0.01*	0.00*	0.00*
21	3	0.00*	0.00*	0.01*	0.21	0.00*
21	4	0.09	0.00*	0.65	0.00*	0.03*
21	5	0.01*	0.00*	0.00*	0.34	0.15

Significant *p* values are indicated with an asterisk.

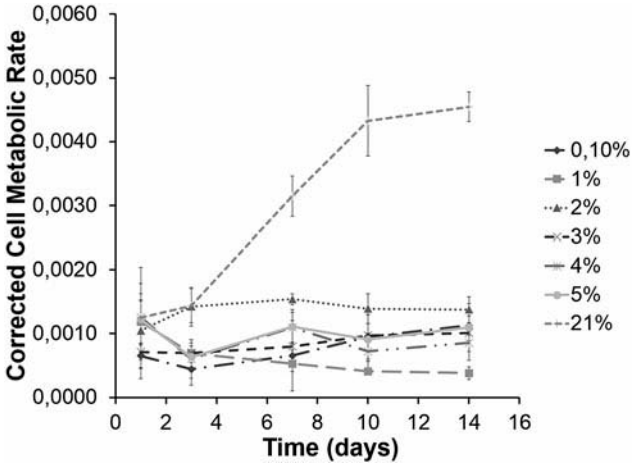

Figure 3. Line graph representing the corrected cell metabolic rate. This was calculated at five time points over 14 days for several oxygen percentages. Error bars represent the standard error of the mean.

Table 3. *P* values of differences in CCMR of 21% oxygen as compared to all other tested oxygen percentages, for five time points.

		Time (days)				
O_2 percentage (%)	Compared to (%)	1	3	7	10	14
21	0.1	0.55	0.08	0.08	0.08	0.08
21	1	0.83	0.05*	0.05*	0.05*	0.05*
21	2	0.83	0.64	0.05*	0.05*	0.05*
21	3	0.27	0.05*	0.05*	0.05*	0.05*
21	4	0.51	0.10	0.05*	0.05*	0.05*
21	5	0.83	0.05*	0.05*	0.05*	0.05*
1	2	0.66	0.13	0.05*	0.05*	0.05*

p values ≤ 0.05 are indicated with an asterisk.

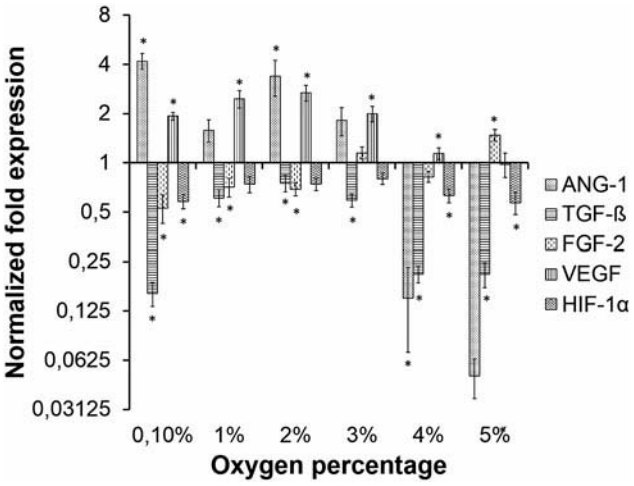

Figure 4. Bar chart representing the normalized fold expression of ANG-1, TGF-β, FGF-2, VEGF, and HIF-1α at several oxygen percentages when compared to expression of these factors at 21% oxygen. Normalized fold expressions that differ significantly from normalized fold expression at 21% oxygen are indicated with an asterisk. Error bars represent the standard error of the mean.

in the 1%–3% oxygen range, ($p < 0.05$), especially at 2% oxygen (See Figure 4). Angiopoetin-1 (ANG-1) had a similar expression profile, but expression was only significantly higher at 2% O_2. There was a significantly lower expression of transforming growth factor-β1 (TGF-β1) and HIF-1α in hMSCs exposed to hypoxic conditions, yet the highest expression of these factors was still found in cells cultured at 2% O_2. The expression of fibroblast growth factor-2 (FGF-2) remained fairly constant under hypoxic conditions. Expression levels of VEGF-receptor2 (VEGF-*R*2) and platelet-derived growth factor-β (PDGF-β) were very low to nihil in hMSCs (data not shown).

Discussion

The first aim of this study was to find the oxygen concentration range at which hMSCs proliferate the most. The second aim was to investigate the expression of AGF by hMSCs in response to decreasing oxygen concentrations.

Human MSC showed the highest proliferation rate when cultured at 1% oxygen. When the cell metabolic rate was corrected for the number of cells, cells cultured at 21% oxygen showed the highest CCMR. The expression of VEGF and ANG-1 in hMSCs was highest at 2% oxygen. Other AGF were underexpressed compared to the AGF expression in hMSCs cultured at 21% oxygen.

Bone marrow-derived stem cells reside in a niche where 1–2% oxygen is available (11,12); this being their natural habitat, it was expected that the highest cell proliferation rates would be found at 1%–2% oxygen. As the oxygen percentage increased, cell proliferation happened at a slower pace. hMSCs do not naturally reside under such high oxygen percentages, and therefore, proliferation may be impaired. Previous researchers found similar results (12,22–24). However, exposing the cells to an even lower oxygen percentage of 0.1% resulted in lower cell proliferation. Potier et al. (18) found that prolonged severe hypoxia resulted in lower cell survival than when cells were exposed to hypoxia for only a short period of time, which corroborates with our findings.

The highest corrected cell metabolic rate was found at 21% O_2. The cell metabolic activity was assayed using an MTT test, which measures the mitochondrial activity within a cell. Oxidative phosphorylation, which is the process of aerobic production of ATP, takes place in the cell mitochondria. We hypothesize that the high amount of oxygen to which cells are exposed, at 21% oxygen, leads to higher oxidative phosphorylation and thus to higher mitochondrial activity in the cells. This hypothesis is supported by the findings of Duguez et al. (25), who exposed myocytes to normoxic and hypoxic conditions. They found that cell mitochondrial activity was higher in cells exposed to normoxia than in cells exposed to hypoxia. Secondly, Grayson et al. (16) and Lavrentieva et al. (26) found that under hypoxic conditions, glucose consumption and lactate production were higher in cells cultured under low oxygen conditions than in cells cultured at 21% oxygen, which indirectly indicates the switch from aerobic to anaerobic metabolism. The mitochondria are thus less activated under hypoxic conditions. The lower mitochondrial activity in cells cultured under hypoxic conditions might result in lower MTT values than those found in cells cultured under normoxic conditions. On the other hand, Weir et al. (27) found that mitochondrial activity of keratinocytes assessed using the MTT assay was not sensitive to oxygen concentration. Apparently, sensitivity to oxygen levels is cell type dependent.

The angiogenic cascade is initiated by stabilization of HIF-1α (6,28–30). Our results support this theory, as expression of HIF-1α neither increased nor decreased under hypoxic conditions. Previous studies corroborate with these results (31), and increased HIF-1α protein levels have been found in cells cultured under hypoxic conditions (15). Some authors nonetheless state that HIF-1α expression is upregulated under hypoxic conditions, as their results with cultured MSCs show (17). In Holzwarth's study, glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as a reference gene, and since GAPDH is active in oxidative phosphorylation, its expression may be effected when cells switch from

aerobic to anaerobic conditions. RPL13a is considered as the reference gene of choice for cells cultured under hypoxic conditions (32).

One of the major angiogenic factors that comes to expression in response to activation of the HRE by binding with the HIF-1α complex is VEGF. The increase in VEGF mRNA quantities after exposure of hMSCs to hypoxic conditions has been described by many other researchers (18,33–35). The other angiogenic factor we found to be overexpressed under hypoxic conditions was ANG-1. The expression of ANG-1 in hMSCs cultured under hypoxic conditions has been studied less frequently, but Hu et al (36). found increased HIF-1α, VEGF, and ANG-1 protein levels in mouse MSCs, which corroborates our results.

In our study, FGF-2 and TGF-β1 generally came to expression less under hypoxic conditions than under normoxic conditions. The AGF primer set was composed aiming at representation of AGF active during all stages of vascular development—initiation of vascular growth, migration, and proliferation of blood vessel cells, and finally maturation of the newly formed blood vessel. Initiation of blood vessel formation is importantly regulated by VEGF (37,38). Perhaps vascular ingrowth is mainly initiated by MSCs and is the process preceded by release of AGF by other cells involved in vascular sprouting (37,38). Release of FGF-2 and TGF-β1 by hMSCs would thus not be necessary, which may explain why FGF-2 and TGF-β1 expressions in hMSCs did not increase.

The occurrence of a peak AGF expression in hMSCs cultured at 2% O_2 may seem remarkable, as this oxygen percentage is the same as in their natural habitat. The implication is that they naturally and continuously produce AGF, but it is not clear yet whether this is the case. This AGF production can be of great value in fracture hematomas, where oxygen tension is very low, around 1% (39). After a fracture, repair cells such as MSCs are attracted toward the fracture hematoma through the release of chemokines, initiating the growth of fresh, vascularized bone tissue (40–42). The presence of AGF-releasing hMSCs in a fracture hematoma at this oxygen percentage may thus support the attraction of cells that form a new vascular supply.

The hMSCs used in this study were isolated under normoxic conditions. It could therefore be that a specific population was selected that is more adapted to living under normoxic conditions than cells which have only been exposed to hypoxic conditions. An effort was made to isolate hMSCs from full bone marrow under hypoxic conditions, but the isolated cells did not reach 70% confluence, even after four weeks of culturing (data not shown). Other authors also report difficulties isolating hMSCs from full bone marrow under hypoxic conditions (17). This is why cells isolated under normoxic conditions were used in the experiments. However, the cells used in our study performed well in terms of cell proliferation, metabolic activity, and AGF production under hypoxic conditions, so the normoxic conditions during isolation do not seem to have influenced the outcomes of culture experiments under hypoxia in a negative way.

As there is variation in hMSC characteristics between different donors, we did not want to add gender as another factor that could influence stem cell characteristics. It was therefore chosen to use bone marrow only from female donors and not from both sexes. The results may be different if the same experiments are performed on hMSCs derived from male donors.

These results indicate that exposing hMSCs to an oxygen level of 1–2% results in the highest cell proliferation and the highest expression of VEGF and ANG-1. This may indicate that at 1%–2% oxygen hMSCs stimulate vascular ingrowth the most, while cell expansion takes place at the highest rate. This optimal oxygen range should be taken into account in future research when aiming at the most effective tissue regeneration.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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